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Original Citation:

Nutritionally relevant concentrations of resveratrol and hydroxytyrosol mitigate oxidative burst of human granulocytes and monocytes and the production of pro-inflammatory mediators in LPS-stimulated RAW 264.7 macrophages / Bigagli, Elisabetta; Cinci, Lorenzo; Paccosi, Sara; Parenti, Astrid; D'Ambrosio, Mario; Luceri, Cristina. - In: INTERNATIONAL IMMUNOPHARMACOLOGY. - ISSN 1567-5769. - ELETTRONICO. - 43:(2017), pp. 147-155. [10.1016/j.intimp.2016.12.012]

Availability:

This version is available at: 2158/1079511 since: 2017-04-19T14:19:49Z

Published version:

DOI: 10.1016/j.intimp.2016.12.012

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Nutritionally relevant concentrations of resveratrol and hydroxytyrosol mitigate oxidative burst of human granulocytes and monocytes and the production of pro-inflammatory mediators in LPS-stimulated RAW 264.7 macrophages.

Elisabetta Bigagli*¹, Lorenzo Cinci¹, Sara Paccosi², Astrid Parenti², Mario D'Ambrosio¹ and Cristina Luceri¹.

¹Department of Neuroscience, Psychology, Drug Research and Child Health - NEUROFARBA – Section of Pharmacology and Toxicology, University of Florence, Viale G. Pieraccini 6, Florence, Italy.

²Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Viale G. Pieraccini 6, Florence, Italy.

*Corresponding author: Elisabetta Bigagli, Dept. NEUROFARBA, University of Florence, Viale Pieraccini 6, 50139 Florence; phone: +39 055 2758303; e-mail: elisabetta.bigagli@unifi.it

Abstract

The health benefits of bio-active phenolic compounds have been largely investigated *in vitro* at concentrations which exceed those reachable *in vivo*. We investigated and compared the anti-inflammatory effects of resveratrol, hydroxytyrosol and oleuropein at physiologically relevant concentrations by using *in vitro* models of inflammation.

Human granulocytes and monocytes were stimulated with phorbol myristate acetate (PMA) and the ability of resveratrol, hydroxytyrosol and oleuropein to inhibit the oxidative burst and CD11b expression was measured. Nitric oxide (NO), prostaglandin E2 (PGE2) levels, COX-2, iNOS, TNF α , IL-1 β and miR-146a expression and activation of the transcription factor Nrf2 were evaluated in macrophages RAW 264.7 stimulated with LPS (1 μ g/ml) for 18 h, exposed to resveratrol, hydroxytyrosol and oleuropein (5 and 10 μ M). Synergistic effects were explored as well, together with the levels of PGE2, COX-2 and IL-1 β expression in macrophages after 6 h of LPS stimulation. PGE2 and COX-2 expression were also assessed on human monocytes.

All the tested compounds inhibited granulocytes oxidative burst in a concentration dependent manner and CD11b expression was also significantly counteracted by resveratrol and hydroxytyrosol. The measurement of oxidative burst in human monocytes produced similar effects being resveratrol more active. Hydroxytyrosol and resveratrol inhibited the production of NO and PGE2 but did not reduce iNOS, TNF α or IL-1 β gene expression in LPS-stimulated RAW 264.7 for 18 h. Resveratrol slightly decreased COX-2 expression after 18 h but not after 6 h, but reduced PGE2 levels

after 6 h. Resveratrol and hydroxytyrosol 10 μ M induced NRF2 nuclear translocation and reduced miR-146a expression in LPS treated RAW 264.7 .

Overall, we reported an anti-inflammatory effect of resveratrol and hydroxytyrosol at low, nutritionally relevant concentrations, involving the inhibition of granulocytes and monocytes activation, the modulation of miR-146a expression and the activation of Nrf2. A regular dietary intake of resveratrol and hydroxytyrosol may be a useful complementary strategy to control inflammatory diseases.

Keywords: inflammation, oxidative burst, resveratrol, hydroxytyrosol, miR-146a, Nrf2

1. Introduction

Human studies demonstrated that a diet rich in phenolic compounds exerts health-promoting effects and reduce inflammatory markers associated to chronic diseases [1-2]. Although the effects of phenolic compounds are mainly ascribed to their direct antioxidant/radical scavenger activity, recent findings indicate that they can also regulate the activity of transcription factors and enzymes and affect the expression of microRNAs (miRNAs) [3-5]. We recently reported that 5 and 1 μ M resveratrol, concentration dependently modulated the expression of thousands of miRNAs in neuroglial co-cultures [6]. Interestingly, the intracellular concentrations required to affect these cell signaling pathways seems to be lower than those required to have antioxidant activity [7] and close to that observed *in vivo*.

Resveratrol, is a natural polyphenol found in grapes, red wine and peanuts with various activities including anti-inflammatory, anti-oxidative, anti-proliferative and chemopreventive effects [8]. There are numerous reports showing that resveratrol can attenuate the release of pro-inflammatory mediators [9-11] in addition to the well documented antioxidant and ROS scavenging activity [12].

More recently, the phenolic fraction of olive oil, mainly comprised of hydroxytyrosol and oleuropein, has also gained attention. Oil phenols are in fact inhibitors of oxidation damage and inflammation *in vitro* and *in vivo* in a variety of biological systems. Hydroxytyrosol is a potent radical scavenger and a chelator of metals [13] and exerts anti-inflammatory effects through COX-2 and iNOS inhibition [14]. Oleuropein is a radical scavenger [15] and can decrease the production of IL-1 β in human cells [16] .

Resveratrol and hydroxytyrosol are absorbed by passive diffusion [17-18] whereas a glucose transporter has been proposed for oleuropein-glycoside [19]. Low micromolar concentrations (1-10 μ M) are achievable in human biological fluids after daily intake of extra-virgin olive oil [20] or after a single oral dose of resveratrol (25 mg) [18]. Olive oil phenolics and their metabolites are distributed to all body and especially to the liver and kidney [21-22]. *In vitro* anti-

inflammatory properties of these compounds have been extensively investigated at very high concentrations (50–200 μ M), which are never reached *in vivo* [23]. Therefore, more studies are needed to validate the biological effects of dietary phenolic compounds in the low micromolar range [24].

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (NRF2), is a master regulator of redox homeostasis [25] but its activation also inhibit pro-inflammatory mediators including cytokines, cyclooxygenase-2 and inducible nitric oxide synthase. Therefore, the identification of new Nrf2-dependent anti-inflammatory phytochemicals has become a key point in drug discovery [26].

MicroRNAs (miRNAs) are small, endogenous noncoding RNA molecules (18–25 nucleotides) that work as posttranscriptional regulators of gene expression by binding to the 3' untranslated region (3' UTR) of target mRNAs [4].

Among the several miRNAs regulating the inflammatory process, the activation of miR-146 during the inflammatory response to LPS was reported in human monocytes [27] and in RAW264.7 macrophage cells [28]. A recent study also showed that miRNA-146a regulated Nrf2 translation through binding to Nrf2 mRNA in aging [29].

In the present study, we therefore investigated and compared the anti-inflammatory effects of resveratrol, hydroxytyrosol and oleuropein at concentration compatible with *in vivo* reachable concentrations and explored the mechanisms involved.

2. Materials and methods

2.1 Chemicals

Resveratrol (with a purity > 99%), hydroxytyrosol (with a purity > 98%), oleuropein (with a purity > 98%) and Indomethacin (with a purity > 99%), were purchased from Sigma-Aldrich.

2.2 Purification of peripheral blood granulocytes

Peripheral blood samples were collected in EDTA vacutainers from 3 healthy volunteers. Granulocytes were prepared from about 7 ml of freshly collected blood samples by a one-step double density centrifugation method [30]. Each whole blood sample was diluted 1:3 with sterile PBS at room temperature (RT) and carefully layered onto a double density gradient prepared by carefully layering 3.5 ml of Ficoll with density 1077 g/l (Lympholyte H, CL5020, Cederlane) over 3.5 ml of Ficoll with density 1113 g/l (Lympholyte poly, CL5070, Cederlane). After centrifugation (500 g for 40 minutes at RT) granulocytes were found at the 1077/1113 interphase and collected by gentle pipetting. In order to eliminate Ficoll (which might interfere with the next step), the suspension was diluted by adding 10 ml of PBS per each ml of suspension. After spinning at 700 g for 5 min at 4°C the supernatant was removed and erythrocytes were

1 lysed in 45 ml of a solution of NH_4Cl 0.157M - KHCO_3 0.01M - EDTA 100 μM at 4°C for 10 min. Granulocytes were
2 then pelleted at 700 g for 5 min at 4°C , re-suspended in 1 ml of PBS, counted and kept in ice until use.
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8 **2.3 Preparation of resveratrol, hydroxytyrosol and oleuropein solutions**

9 Resveratrol, hydroxytyrosol and oleuropein were dissolved in DMSO, ETOH and PBS, respectively, and diluted in
10 complete cell culture medium in order to obtain the appropriate concentrations to be tested. The final concentrations of
11 DMSO and ETOH were less than 0.1%. Indomethacin, was dissolved in PBS.
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17 **2.4 Oxidation of luminol by granulocytes-generated reactive oxygen species (ROS)**

18 Granulocytes oxidative burst was measured by chemiluminescence, by monitoring ROS-induced oxidation of luminol,
19 according to a previously described procedure [31] with few modifications. Reaction mixtures contained, in a final
20 volume of 200 μl , the following reagents at the indicated final concentrations: neutrophils (10^6 cells/ml), resveratrol,
21 hydroxytyrosol, oleuropein and indomethacin at various concentrations (0–100 μM), luminol (500 μM), and PMA (10^{-7}
22 M). Cells and tested compounds were pre-incubated for 5 min at 37°C before the addition of luminol and PMA. The
23 measurements were carried out at 37°C . Kinetic readings were initiated immediately after cell stimulation.
24 Measurements were taken at the peak of the curve. In all experiments, this peak was observed around 5 min. The effects
25 are expressed as percentage of inhibition of luminol oxidation. Experiments were performed in triplicate.
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37 **2.5 Measurement of oxidative burst in monocytes**

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39 Two ml of fresh peripheral venous blood, collected in EDTA vacutainers, were haemolysed by the addition of 48 ml of
40 lysis solution (NH_4Cl 0.157M - KHCO_3 0.01M - EDTA 100 μM). The suspension was incubated for 5 min at 4°C and
41 then centrifuged at 300 g for 5 min at 4°C . Pellet was suspended in 2 ml PBS at 4°C supplemented with 0.9 mg/ml
42 glucose (PBS–glucose) for monocyte's oxidative burst analysis. The samples were kept on ice and the experiments were
43 performed within 30 min to avoid further cell activation.
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50 Oxidative burst was performed according to [32] with some modifications. The fluorescence generated in monocytes by
51 the oxidation of the 2',7'-Dichlorofluorescein diacetate (DCFH-DA) probe (Molecular Probes, Eugene, Oregon, USA)
52 with hydrogen peroxide was quantified by means of flow cytometry (Facs CantoII, BD). Leucocytes (10^6 cells/ml),
53 were incubated with PBS–glucose supplemented with 5 μM of DCFH-DA for 15 min in 37°C . Cells were pre-incubated
54 or not (positive control) for 5 min at 37°C with resveratrol, hydroxytyrosol or oleuropein at 5 or 10 μM at RT. The
55 H2O2 production during cellular activation was determined by adding 10^{-7} M phorbol-12-myristate 7-acetate (PMA)
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(Sigma) for 15 min at 37°C. Cells not pre-treated with test substances or PMA and incubated in PBS–glucose for 15 min at 4°C served as negative control. Activation was determined by the addition of 1 ml PBS supplemented with 2.5 mM EDTA pH 7.2 and 0.1% FBS. Cells were then subjected to flow cytometry, monocytes were gated on forward light scattering/ sideways light scattering (FSC/SSC) and data were acquired as Mean Fluorescence Intensity (MFI) and expressed as percentage of the oxidative burst inhibition vs positive control.

2.6 Analysis of CD11b granulocytes expression

In order to study the expression and ability to mobilize the adhesion molecule CD11b, leucocytes isolated from peripheral circulation (106 cells/ml) were pre-incubated or not (positive control) for 5 min at 37°C in RPMI-1640 with 5% FBS with resveratrol, hydroxytyrosol or oleuropein at 5 or 10 µM at RT. Cells were then incubated for 15 min at 4°C or not (negative control) with 10⁻⁷ M PMA for 15 min at 37°C. Then cells were washed once in PBS–azide (300 g for 5 min, 4°C). The expression of CD11b was analyzed by the addition of 5 µl of PE-conjugated monoclonal anti-CD11b (BD Pharmigen, Los Angeles, CA). PE Mouse IgG2a, κ Isotype Control (BD Pharmigen) was used to determine non-specific binding. Leucocytes were incubated with antibodies for 30 min 4°C, washed once in 3 ml cold PBS–azide and finally suspended in 0.3 ml 4°C PBS–azide before analysis by flow cytometry. Granulocytes were selected in a FSC/SSC dot plot according to their dimension and complexity [33].

2.7 Cell culture

RAW264.7, a murine macrophage cell line, (kindly provided by Prof. Bani, University of Florence and previously obtained from American Type Culture Collection (Rockville, USA) was cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C in an atmosphere containing 5% CO₂.

2.8 Lipopolysaccharide (LPS) induced inflammation in RAW264.7

RAW 264.7 cells were cultivated in a 24-well microplate (1 × 10⁶ cells/well) and treated with 5-10 µM resveratrol, hydroxytyrosol and oleuropein or with indomethacin 10 µM mixed with LPS (1 µg/ml) for 6 h or 18 h at 37 °C. In order to explore potential synergistic effects, RAW 264.7 cells were also treated with hydroxytyrosol 10 µM mixed with resveratrol 1 µM in the presence of LPS (1 µg/ml) for 18 h at 37 °C. At the end of treatments, cell lysates were collected for genes and microRNA expression analyses and supernatants for nitric oxide and PGE₂ determination.

2.9 LPS induced inflammation in human monocytes

Human monocytes were isolated with CD14+ MicroBeads (Miltenyi Biotec, Germany) according to manufacturer's instructions. 2×10^5 monocytes were treated with 5-10 μ M resveratrol, hydroxytyrosol or oleuropein mixed with LPS (1 μ g/ml) for 18 h at 37 °C. At the end of treatments, cell lysates were collected for genes expression analyses and supernatants for PGE2 determination.

2.10 Determination of nitric oxide (NO) production

The anti-inflammatory activity of resveratrol, hydroxytyrosol and oleuropein was determined on the basis of NO production in macrophage culture supernatants using the Griess reaction [34]. RAW 264.7 cells (kindly provided by Prof. Bani, University of Florence and previously obtained from American Type Culture Collection (Rockville, USA)) were cultivated in a 24-well microplate (1×10^6 cells/well, in DMEM medium without phenol red containing 10% FBS. Cells were incubated at 37 °C in a 5% CO₂ incubator until confluence. The cultured cells were treated with resveratrol, hydroxytyrosol and oleuropein at 5 and 10 μ M and with indomethacin 10 μ M mixed with lipopolysaccharide solution (LPS, 1 μ g/ml). After incubating for 18 h at 37 °C, the cultured cell supernatant (100 μ l) was mixed with an equal volume of Griess reagent (1% [w/v] sulfanilamide and 0.1% [w/v] N-[1-naphthyl] ethylenediamine hydrochloride in 2.5% [v/v] phosphoric acid) and incubated at RT for 10 min. The absorbance was measured at 540 nm using a microplate reader. NO production was calculated with reference to a standard curve obtained with NaNO₂. Experiments were performed in triplicate.

2.11 Prostaglandin E2 (PGE2) determination

PGE2 levels were measured in the supernatants using an immune-enzymatic method (Cayman) according to the manufacturer's specifications. Indometacin 10 μ M, was used as positive control.

2.12 RT-PCR

Total RNA from RAW 264.7 cells was isolated using the miRNeasy Mini Kit (Qiagen Duesseldorf, Germany). For first-strand cDNA synthesis, 1 μ g of total RNA from each sample was reverse-transcribed. Primers were designed on the basis of the mouse GenBank sequences for cyclo-oxygenase-2 (COX2), inducible NO synthase (iNOS), IL1 β and TNF α . Ribosomal protein large P1 (RPLP-1) was co-amplified as the reference [35].

2.13 miR-146a expression by Quantitative Real-Time PCR (qRT-PCR).

Reverse transcription of RNA was performed using the miScript Reverse Transcription Kit according to manufacturer's instructions (Qiagen). The miScript PreAMP PCR Kit was used for the pre-amplification step before qRT-PCR of

miRNA. qRT-PCR assays were carried out in 7900HT Fast Real-Time PCR System (Applied Biosystems) using miScript SYBR Green PCR Kit and miScript Primer Assay according to manufacturer's instructions (Qiagen). Briefly, each reaction was performed in a final volume of 25 µl containing 2,5 µl of the cDNA, a master mix containing 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix, 2.5 µl of 10x miScript Universal Primer, 2.5 µl of 10x miScript Primer Assay and RNase-free water. The amplification profile was denaturation at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30s, and 70°C for 30s. The expression levels of miR-146a were normalized to RNU6B and calculated as $2^{-\Delta\Delta Ct}$.

2.14 Immunocytofluorescence for Nrf2 localization

RAW 264.7 cells were grown in poly-D-lysine-coated glass dishes for 24 h, then treated with LPS (1 µg/ml), and tested compounds for 18 h. Cells were then fixed with cold 4% (w/v) paraformaldehyde for 20 min, rehydrated in PBS for 15 min, and permeabilized in 0.1% (w/v) TritonX-100 at RT for 10 min. After being washed with PBS, the cells were blocked for unspecific fluorescence with 3% BSA for 1 hour and then incubated with Rabbit anti-Nrf2 polyclonal antibody C-20 (1:300) (Santa Cruz Biotechnology) at 4°C overnight followed by the fluorescent secondary antibodies: AlexaFluor 488 goat anti-mouse (1:333) (Invitrogen). Cells were also counterstained with DAPI dye to show the nuclear morphology.

2.15 Western blotting for Nrf2 in cytoplasmic and nuclear extracts

RAW 264.7 cells (1×10^6 cells) were seeded in T-25 flasks and allowed to adhere for 24 h. After being treated with LPS and the tested compounds, the cells were collected by trypsinization. Nuclear and cytoplasmic extractions were performed using the Qproteome Cell Compartment kit (Qiagen) following manufacturer's instructions. Protein content was estimated by using the Bio-Rad DC protein assay kit (Bio-Rad, Segrate, Milan, Italy). Thirty micrograms of protein were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation (NuPAGE, Novex) and transferred to iBlot 2 Nitrocellulose membranes by using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific). Immunostaining was performed with Rabbit anti-Nrf2 polyclonal antibody C-20 (1:100) (Santa Cruz Biotechnology) followed by incubation with anti-rabbit IgG horseradish peroxidase-linked antibody (Cell Signaling), 1:4,000 for 1 hour at RT. Proteins were visualized using the enhanced chemiluminescence procedure with Immobilon Horseradish Peroxidase Substrate (Millipore) and immune-reactive bands were acquired through the Image Quant 350 (GE Healthcare Life Sciences, Buckinghamshire, UK) and quantified by densitometric analysis using the Quantity-One software (Bio-Rad Laboratories, Hercules, CA). Each density measure in the nuclear extracts was normalized to anti-

Histone H3 Rabbit antibody (Cell Signaling) (1:1000) and glyceraldehyde 3-phosphate dehydrogenase (14C10) rabbit antibody (Cell Signaling), (1:3,000) level as internal control for cytoplasmic extracts.

2.16 Image Acquisition and analysis

Microscopic analysis were performed with a fluorescence microscopy (Labophot-2, Nikon) connected to a CCD camera. Ten photomicrographs were randomly taken for each sample (average number of cells/field: 100; average number of analyzed cells in total: 1000) and the number of cells with Nrf2 nuclear translocation was counted using ImageJ 1.33 image analysis software (<http://rsb.info.nih.gov/ij>).

2.17 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Newman-Keuls post hoc test when normally distributed or using the non-parametric Kruskal–Wallis with Dunn's post hoc test. All analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Results are expressed as means \pm standard error (SEM) of three independent experiments. P value of 0.05 was considered significant.

3. Results

3.1 Effect of resveratrol, hydroxytyrosol and oleuropein on ROS production by human granulocytes and monocytes stimulated with PMA

Resveratrol, hydroxytyrosol and oleuropein decreased the levels of ROS at all concentration tested (Figure 1, panel A).

This effect was concentration-dependent and already evident at concentrations as low as 5 μ M. Resveratrol and hydroxytyrosol at the highest concentrations (25-50-100 μ M) inhibited 60-80% of ROS-induced oxidation of luminol. Oleuropein displayed the smallest inhibitory activity, reaching 50% of inhibition of ROS-induced oxidation of luminol only at 100 μ M. Interestingly, hydroxytyrosol demonstrated an higher potency, counteracting ROS production by 27 and 37% even at the lowest concentrations tested (10 and 5 μ M). At 10 μ M, resveratrol and oleuropein inhibited ROS production by 15%, whereas the effect of resveratrol 5 μ M was negligible. Indomethacin 100 and 50 μ M reduced ROS-induced oxidation of luminol by 40% but at 10 and 5 μ M, was not effective.

To confirm the anti-inflammatory effect of test substances, the up-regulation of CD11b was assessed on PMA-stimulated granulocytes. PMA significantly stimulated CD11b expression within 15 minutes ($P<0.001$), which was prevented by hydroxytyrosol 5 and 10 μ M and significantly inhibited by 5 μ M resveratrol (76% inhibition, $P<0.01$, Figure 1, panel B). Oleuropein was devoid of any effect.

The effect on ROS inhibition was also tested on human monocytes stimulated with PMA. Resveratrol was the most active in inhibiting ROS production, with 10 μ M producing an inhibition of luminol oxidation by $32 \pm 8\%$ (Figure 1, panel C). The inhibition of ROS production by hydroxytyrosol was weaker than that of resveratrol but statistically significant, while oleuropein was ineffective.

3.2 Effect of resveratrol, hydroxytyrosol and oleuropein on NO production by RAW 264.7 macrophages stimulated with LPS

LPS significantly stimulated NO production by RAW 264.7 cells ($p < 0.001$) (Figure 2). This effect was significantly decreased by resveratrol at 10 and 5 μ M ($p < 0.01$). Hydroxytyrosol exhibited a comparable and concentration-dependent inhibitory effect on LPS-induced NO production, with a significant reduction of NO at 5 ($p < 0.05$) and 10 μ M ($p < 0.01$). On the contrary, oleuropein did not exert any significant effect (Figure 2). In this model, indomethacin 10 μ M was not effective since NO levels were similar to those observed in untreated, LPS stimulated cells ($48 \pm 7 \mu$ M vs $43 \pm 2 \mu$ M).

3.3 Effect of resveratrol, hydroxytyrosol and oleuropein on COX-2, iNOS, TNF α and IL1 β expression in RAW 264.7 macrophages and human monocytes stimulated with LPS

Resveratrol 5 and 10 μ M significantly attenuated the LPS-induced expression of COX-2 after 18 h and this effect was independent on the concentration ($p < 0.05$). On the contrary, hydroxytyrosol and oleuropein were unable to inhibit COX-2 expression (Table 1). Upon LPS stimulation for 18 h, the expression of IL-1 β , iNOS and TNF α was also significantly induced in RAW 264.7 macrophages but none of the phenolic compounds analyzed, exerted significant effects (Table 1). We also investigated the effects of the tested compounds on LPS stimulated RAW 264.7 after 6 hours. LPS significantly induced the expression of COX-2 (0.27 ± 0.02 vs 0.15 ± 0.01 ; $p < 0.05$) and IL1 β (0.78 ± 0.05 vs 0.53 ± 0.04 ; $p < 0.05$) in RAW 264.7 and the expression of COX-2 (0.79 ± 0.07 vs 0.42 ± 0.04 ; $p < 0.05$) and IL1 β (0.93 ± 0.04 vs 0.58 ± 0.05 ; $p < 0.05$) compared to control unstimulated monocytes, but the tested substances, did not exert significant effects (data not shown).

3.4 Effects of resveratrol, hydroxytyrosol and oleuropein on LPS induced PGE2 production in RAW 264.7 macrophages and human monocytes

After 18 h of LPS stimulation, in the PGE2 assay, as expected, indomethacin 10 μ M completely suppressed PGE2 production. Similarly, resveratrol concentration-dependently suppressed PGE2 production by 85 %; hydroxytyrosol was effective at 10 μ M (-38% ; $p < 0.05$) but not at 5 μ M. Resveratrol 1 μ M was not effective in reducing PGE2 levels;

1 however, when a mixture containing hydroxytyrosol 10 μ M and resveratrol 1 μ M was tested, PGE2 levels were
2 reduced by 48% ($p < 0.05$ vs LPS) indicating a weak, even if not significant, synergistic effect compared to
3 hydroxytyrosol 10 μ M alone. Oleuropein did not exert any significant effect (Figure 3, panel A). Resveratrol 5 and 10
4 μ M was also able to significantly ($p < 0.01$) reduce PGE2 levels by 66 and 81%, respectively, at an earlier time point (6
5 h after co-treatment with LPS). On the contrary, neither hydroxytyrosol nor oleuropein affected PGE2 levels after 6 h
6 (Figure 3, panel B). In human monocytes, 5 and 10 μ M resveratrol and hydroxytyrosol, significantly decreased PGE2
7 production by nearly 50% and by 40% respectively, whereas oleuropein exerted a weak effect (-12%) only at 10 μ M
8 (Figure 3, panel C).

18 **3.5 Resveratrol and hydroxytyrosol but not oleuropein, induced the nuclear translocation of Nrf2 and decreased** 19 **the expression of miR-146a in LPS-stimulated macrophages.**

22 Nrf2 translocation from the cytoplasm to the nucleus is an indispensable step to study Nrf2 activation (Figure 4, panel
23 A-E). The percentage of cells with nuclear Nrf2 staining in LPS treated cells was significantly reduced compared to
24 control group (77.7 ± 3.3 vs. 51.7 ± 8.9) ($p < 0.05$). On the contrary, treatment with resveratrol and hydroxytyrosol
25 restored such percentage at levels even above those of the control cells (79.3 ± 3.3 and 81.8 ± 1.7 , respectively)
26 ($p < 0.05$) (Figure 4, panel F). Western blotting results confirmed the results obtained by immunocytochemistry
27 demonstrating a decreased ratio between nuclear Nrf2 to cytoplasmic Nrf2 protein expression in cells treated with LPS
28 compared to control cells ($p < 0.05$) that was prevented in those treated with resveratrol ($p < 0.05$) but not with oleuropein.
29 Western blotting results even exalted the effect of hydroxytyrosol which completely prevented the decrease in the ratio
30 between nuclear Nrf2 to cytoplasmic Nrf2 protein expression observed in cells treated with LPS alone ($p < 0.01$) (Figure
31 4, panel G).

34 As shown in Figure 5, the expression of miR-146a was upregulated in LPS-stimulated RAW264.7 macrophages
35 compared to unstimulated cells. Resveratrol and hydroxytyrosol 10 μ M significantly decreased miR-146a expression
36 ($p < 0.05$) whereas oleuropein exerted a similar but not significant effect. Interestingly, we observed an inverse
37 correlation between the global expression of Nrf2 and miR-146a in unstimulated cells, and in those treated with LPS
38 alone or in the presence of resveratrol.

4. Discussion

For several decades, numerous *in vitro* investigations demonstrated that dietary polyphenols attenuate inflammatory responses [36, 14]. Since physiological concentrations do not exceed 10 μ M, the effects of polyphenols *in vitro* at high concentrations, up to 200 μ M, are generally irrelevant. Therefore, we investigated the potential anti-inflammatory effects of three dietary phenols at physiologically relevant concentrations, using *in vitro* models of inflammation.

The first model mimics the acute phase of inflammation in which granulocytes produce excessive amount of ROS causing tissue-damaging inflammatory reactions. We demonstrated that resveratrol, hydroxytyrosol and oleuropein, in a concentration-dependent manner, starting at 5 μ M, inhibited oxidative burst in human granulocytes suggesting a specific biological effect. For the activation of granulocytes, we used PMA which stimulates ROS generation by direct activation of protein kinase C (PKC). The chemiluminescent emission of luminol was applied to measure both intra and extra-cellular ROS since this probe is capable of penetrating cell membranes [37]. Resveratrol, hydroxytyrosol and oleuropein effectively suppressed intra- and extracellular generated ROS. At high concentrations, the efficacy of resveratrol and hydroxytyrosol was comparable but stronger than that of oleuropein; however, at physiologically relevant concentrations (5-10 μ M) hydroxytyrosol was the most potent in decreasing ROS formation. Similarly, Visioli et al. [38] reported that hydroxytyrosol exhibited a strong radical scavenger activity than oleuropein. The presence of ortho-dyphenols in hydroxytyrosol seems confer a more potent radical scavenger activity than simple phenols and the addition of the elenoic acid seems to reduce this property. Similarly, it has been reported that hydroxytyrosol and resveratrol, 10 μ M, inhibited ROS production in human monocytes [39] and neutrophils [40].

This effect can be attributed to both a direct ROS scavenging effect and to an inhibition of ROS production by these compounds; in fact, resveratrol has been reported to serve as a scavenger of ROS [41] but also to decrease the activation of PKC α,β indicating interference with PMA activation of the NADPH oxidase system responsible for oxidative burst in neutrophils [42, 40]. The inhibitory effect on PKC activation and the reduction of NADPH oxidase activity by hydroxytyrosol, has been also recently reported [36,39] in addition to direct scavenging abilities [43].

Thus, both a direct radical scavenger effect or an inhibitory effects on ROS production could be involved in the effects observed in our experimental system. For acute activation of granulocytes, we also looked at an additional parameter, the up regulation of CD11b, an integrin family member mediating the inflammatory response. In this assay, both resveratrol and hydroxytyrosol, but not oleuropein, blunted the PMA induced expression of the adhesion molecule CD11b, suggesting that this molecules may lead to a reduction in the invasive ability of granulocytes. Similar results were obtained by Park et al. (2016) [44] with resveratrol 50 μ M whereas no data are currently available for hydroxytyrosol.

The relevance of these results was further confirmed in an assay employing human monocytes; in this test, we used DCFH-DA which similarly to luminol, can cross cell membranes. We showed that resveratrol and to a lower extent hydroxytyrosol, abrogated this reaction, suggesting their ability to react with intracellular H₂O₂. O'Dowd and coworkers (2004) [43] previously reported a significant effect of hydroxytyrosol 13 μ M in the same model.

We also used LPS-treated RAW 264.7 cells as a model to study late inflammatory responses. After 18 h of co-treatment, resveratrol and hydroxytyrosol 10 μ M, attenuated the LPS-induced production of NO but failed to inhibit iNOS expression, suggesting that NO reduction was not a consequence of a direct enzyme inhibition in the late stage of inflammation. Accordingly, Nosál' et al. [40] reported that iNOS expression was significantly inhibited by resveratrol at 100 μ M and not at 10 μ M; however, at this concentration, it significantly reduced NO production. It is possible that hydroxytyrosol and resveratrol when added to the cells with LPS challenge, failed to inhibit iNOS enzyme induced meanwhile. Pre-treatment studies are generally used for the *in vitro* evaluation of the preventive effects of natural substances but are less indicative of their protective effects. The co-treatment and timing protocol adopted by us together with the low concentrations tested in our study, might explain the differences from other reports [45]. Djoko et al. [11] also suggested that the inhibitory effect on NO production could be mainly due to a post-translational inhibition of iNOS. The lack of efficacy of oleuropein is not surprising since oleuropein even enhanced nitrite production in LPS-challenged mouse macrophages at similar concentrations [46].

Previously, it was shown that resveratrol significantly attenuated LPS-induced PGE₂ and COX-2 in RAW 264.7 at similar concentrations [10]; data on hydroxytyrosol are more contradictory, since some authors reported inhibition of PGE₂ only at high concentrations (50-100 μ M) [47, 36] whereas others, in agreement with us, reported significant effects even at 10-20 μ M [48]. Oleuropein failed to counteract the increase in inflammatory markers tested, contradicting the data recently reported by Ryu et al. in the same model [49]; however, these authors used concentrations of 100-300 μ M which are much higher than those used by us and largely exceed the physiological range.

Moreover, as these natural bioactive compounds are frequently present in the same products, we also looked at potential synergistic effects by combining very low concentration of the most active compound resveratrol, with hydroxytyrosol. Even if the reduction of PGE₂ expression was similar to that observed in hydroxytyrosol treated cells, a weak synergistic effect was detectable since resveratrol alone did not modify PGE₂ levels at all. This is the first attempt to explore the effects of a combination of these two compounds at low concentrations which deserves to be further studied in light of their co-occurrence in health promoting foods.

We used the same model for exploring the early response of macrophages to LPS stimulation; interestingly, resveratrol was the sole compound able to reduce PGE₂ within 6 hours at levels comparable to that at 18 hours indicating that the

kinetic of PGE2 inhibition reaches the maximum within 6 hours and it is sustained at least until 18 hours. On the contrary, hydroxytyrosol showed a different kinetic being able to blunt PGE2 production only after 18 hours. We can suggest that the structural differences confer similar biological properties to hydroxytyrosol and resveratrol but a distinct timing of effects.

The relevance of our findings for human consumption, were further confirmed in human monocytes stimulated with LPS in which resveratrol and hydroxytyrosol were shown to be the most active compounds.

As our results showed that resveratrol and hydroxytyrosol reduced LPS-mediated PGE2 production more effectively than they reduced COX-2 at mRNA level, we hypothesized that a mechanism at least partially independent of the regulation of COX-2 expression could be involved. We therefore investigated the contribution of the intracellular redox-sensitive transcription factor Nrf2, which is essential for the protection against oxidative stresses but has also been known to attenuate inflammation [25;50,51]. Resveratrol was shown to induce Nrf2 nuclear accumulation in cardiomyocytes and recently, α -Viniferin, an analogue of resveratrol, inhibited NO and PGE2 production through activation of Nrf2 in LPS-simulated microglial cells [52-53]. We thus hypothesized that Nrf2 could participate in resveratrol and hydroxytyrosol mediated inhibition of LPS-induced inflammatory responses in macrophages. The inhibition of NO and PGE₂ production by resveratrol and hydroxytyrosol was associated to Nrf2 nuclear translocation, which is indicative of its activation. Subsequent western blotting analysis, confirmed that resveratrol and hydroxytyrosol activated Nrf2 nuclear translocation, an effect that may have contributed to decrease pro-inflammatory mediators.

It has been also recently proposed that the protective properties of phenolic compounds may arise from their ability to modulate miRNAs [3-5]. We also explored the effects of resveratrol, hydroxytyrosol and oleuropein on miR-146a, the expression of which is known to be increased in response to pro-inflammatory stimuli such as LPS [54]. The activation of miR-146 during the inflammatory response to LPS was reported in human monocytes [27]. In our experimental condition, mir-146a was upregulated in response of LPS as suggested by others in both RAW 264.6 and THP-1 cells [28, 55].

In response to inflammatory cues such LPS, miR-146a was induced as a negative-feedback regulator being able to target the adaptor molecules IRAK-1 and TRAF6 downstream are two of the top targets for miR-146a [27]. However, Tsai et al. (2012), [56] showed that LPS-induced miR-146a expression in RAW 264.6 cells was inhibited by Calophyllum inophyllum L. extract, an antiinflammatory agent able to reduce LPS induced COX-2 and nitric oxide production. Similarly, our results suggest that, upon resveratrol and hydroxytyrosol treatment, the negative feedback regulation exerted by miR-146a is no longer necessary since inflammation is already limited in the presence of these

substances. This could therefore represents a new molecular mechanism associated to their anti-inflammatory effects. The unexpected reduction of mir-146a expression in oleuropein treated cells was not associated to a reduction of any pro-inflammatory mediators, hence it is possible that other miR-146a gene targets could explain this finding. Previous data also indicated that miRNA-146a, binds Nrf2 mRNA [29] (Smith et al., 2015); accordingly, we noted a decline in total nuclear and cytoplasmic Nrf2 protein expression in LPS treated cells, that was prevented by resveratrol suggesting a potential cross talk between miR-146a and Nrf2 which deserves further investigations.

5. Conclusions

Collectively, our data demonstrate that resveratrol and hydroxytyrosol and to a lower extent oleuropein, are useful for the pharmacological modulation of ROS production by activated granulocytes and monocytes in the early phase of inflammation. Resveratrol and hydroxytyrosol at nutritionally relevant concentrations are also able to decrease the inflammatory response in macrophages. Even if our results do not allow to demonstrate a direct cause-effect relationship, they provide evidence of a potential involvement of Nrf2 and miR-146a in the anti-inflammatory properties of resveratrol and hydroxytyrosol.

These effects are consistent with the ability of polyphenol-rich diets to decrease inflammatory markers in humans [1-2] and suggest that a regular dietary intake of resveratrol and hydroxytyrosol may be a complementary strategy for the control of inflammatory conditions.

Conflict of interest

The authors declare no conflict of interest

Acknowledgments

The authors are very grateful to Prof. Piero Dolaro for critical reading of the manuscript and his useful suggestions. This project was financially supported by the University of Florence, Fondi di Ateneo Luceri.

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Table 1 Effect of resveratrol, hydroxytyrosol and oleuropein on Cox-2, iNOS, TNF α and IL1 β mRNA expression in RAW 264.7 macrophages stimulated with LPS 1 μ g/mL for 18 h.

	Control	LPS	Resv 5 μ M	Resv 10 μ M	Hyd 5 μ M	Hyd 10 μ M	Oleo 5 μ M	Oleo 10 μ M
Cox-2	0,20 \pm 0,05	0,99 \pm 0,01 **	0,75 \pm 0,01 #	0,79 \pm 0,01#	0,97 \pm 0,08	0,84 \pm 0,08	0,92 \pm 0,06	1,07 \pm 0,09
iNOS	0,28 \pm 0,02	0,94 \pm 0,11 **	1,04 \pm 0,07	0,96 \pm 0,13	0,96 \pm 0,04	0,98 \pm 0,07	1,01 \pm 0,07	1,00 \pm 0,14
IL-1 β	0,39 \pm 0,09	0,95 \pm 0,01 **	0,98 \pm 0,04	0,89 \pm 0,04	0,94 \pm 0,03	0,88 \pm 0,02	0,96 \pm 0,01	0,93 \pm 0,03
TNF- α	0,37 \pm 0,01	0,95 \pm 0,02 **	0,79 \pm 0,12	0,99 \pm 0,03	0,94 \pm 0,02	0,92 \pm 0,03	1,00 \pm 0,05	0,99 \pm 0,02

**P<0.01 vs control cells; # P<0.05 vs LPS

Legends to figures

Figure 1. Concentration-dependent effect of resveratrol, hydroxytyrosol and oleuropein on ROS-induced oxidation of luminol in A) human granulocytes and C) human monocytes stimulated with PMA. Data are expressed as percentage of inhibition of PMA stimulated cells; B) Flow cytometry for CD11b expression in human granulocytes stimulated with phorbol myristate (PMA). Median Florescence Intensity (MFI). Resveratrol 5-10 μ M (R5, R10), hydroxytyrosol 5-10 μ M (H5, H10) and oleuropein 5-10 μ M (O5, O10). Data are expressed as mean \pm S.E.M of three independent experiments. * P<0.05; ** P<0.01; *** P<0.001 vs PMA alone. ^^^ P<0.001 vs control unstimulated cells.

Figure 2. Effect of resveratrol (A), hydroxytyrosol (B) and oleuropein (C) on nitrites production in RAW264.7 macrophages stimulated with LPS 1 μ g/mL for 18 h. Resveratrol 5-10 μ M (R5, R10), hydroxytyrosol 5-10 μ M (H5, H10) and oleuropein 5-10 μ M (O5, O10). Data are expressed as mean \pm S.E.M of three independent experiments. ^^^ P<0.001 vs unstimulated control cells; * P<0.05 vs LPS treated cells; ** P<0.01 vs LPS treated cells.

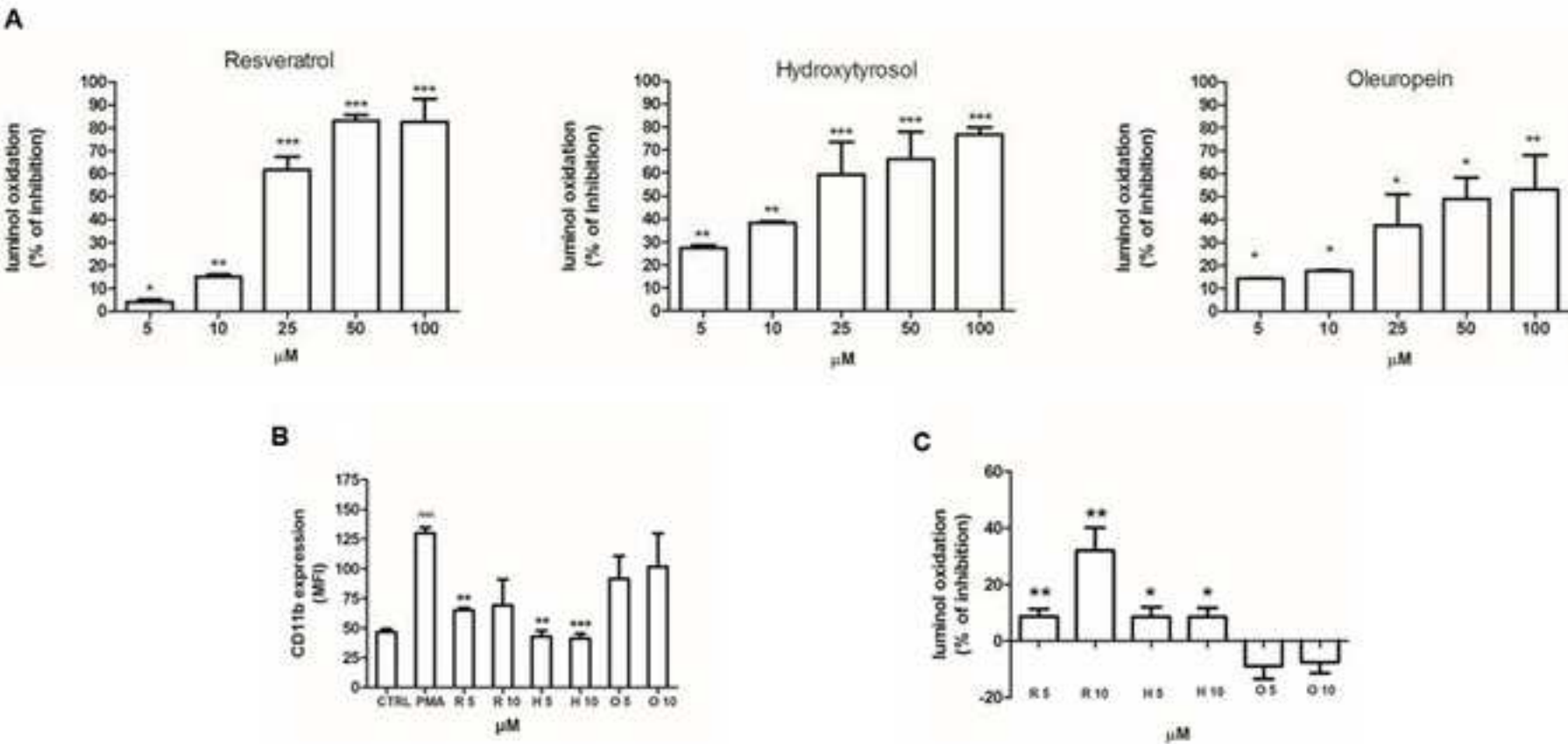
Figure 3. A) Percentage of PGE2 reduction in RAW 264.7 macrophages stimulated with LPS 1 μ g/mL for 18 h and treated with resveratrol 1-5-10 μ M (R1, R5, R10), hydroxytyrosol 5-10 μ M (H5, H10) oleuropein 5-10 μ M (O5, O10) and in those treated with a mixture of hydroxytyrosol 10 μ M and resveratrol 1 μ M (H10+R1). Indometacin (INDO) 10 μ M was used as a positive control. B) Percentage of PGE2 reduction in RAW 264.7 macrophages stimulated with LPS 1 μ g/mL for 6 h and treated with resveratrol 5-10 μ M (R5, R10), hydroxytyrosol 5-10 μ M (H5, H10) and oleuropein 5-10 μ M (O5, O10). C) Percentage of PGE2 reduction in human monocytes stimulated with LPS 1 μ g/mL for 18 h and treated with resveratrol 5-10 μ M (R5, R10), hydroxytyrosol 5-10 μ M (H5, H10) and oleuropein 5-10 μ M (O5, O10). Data are expressed as mean \pm S.E.M of three independent experiments. ** P<0.01 vs LPS treated cells; * P<0.05 vs LPS treated cells.

Figure 4. Localization of Nrf2 in unstimulated RAW 264.7 macrophages (CTRL) (A), in those stimulated with LPS 1 μ g/mL for 18 h alone (LPS) (B) and in those treated with 10 μ M resveratrol (C), hydroxytyrosol (D) and oleuropein (E) (R10, H10, O10). The translocation of Nrf2 was immunostained with Nrf2 antibody (red). DAPI was used to visualize the nuclei (blue) and the merged Nrf2 (red)/DAPI (blue) image is shown in dark pink. Top right Inserts show higher magnification of illustrative cells.

(F): Graph shows quantitative data obtained as follows: ten photomicrographs were randomly taken for each sample (average number of cells/field: 100; average number of analyzed cells in total: 1000). Each bar represents the mean \pm SEM of the percentage of cells positive for Nrf2 nuclear staining/total cells per microscopic field. (G): wester blotting results: ratio between nuclear Nrf2 to cytoplasmic Nrf2 protein expression observed in control cells (CTRL) in those stimulated with LPS 1 μ g/mL for 18 h alone (LPS) and in those treated with 10 μ M resveratrol (R10), hydroxytyrosol (H10) and oleuropein (O10). Each bar represents the mean \pm SEM of three independent experiments. ^ P<0.05 vs control cells; * P<0.05 vs LPS treated cells.

Figure 5. MiR-146a expression in unstimulated RAW 264.7 macrophages (CTRL), in those stimulated with LPS 1 µg/mL for 18 h alone (LPS) and in those treated with 10 µM resveratrol, hydroxytyrosol and oleuropein (R10, H10, O10). Each bar represents the mean \pm SEM of three independent experiments. ^ P<0.05 vs CTRL treated cells; * P<0.05 vs LPS treated cells.

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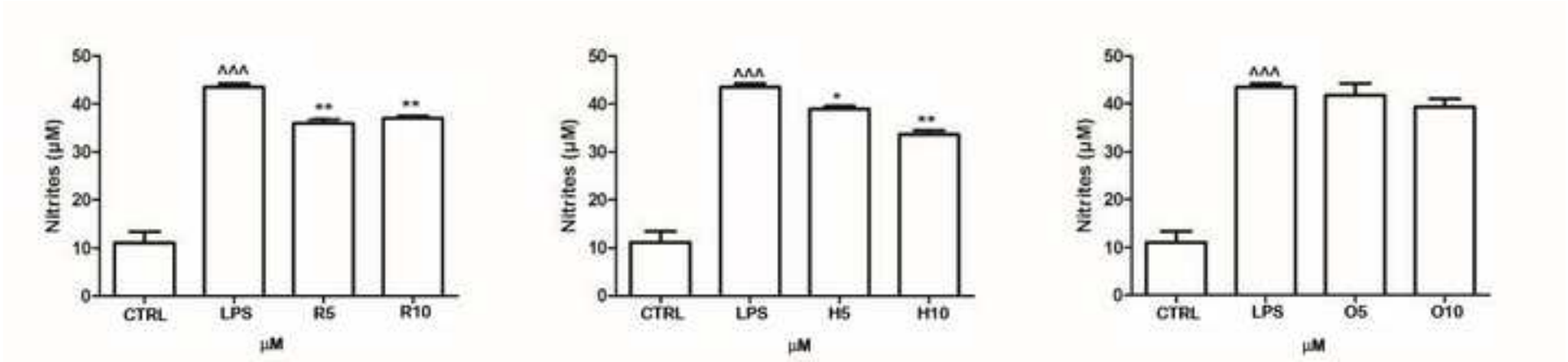


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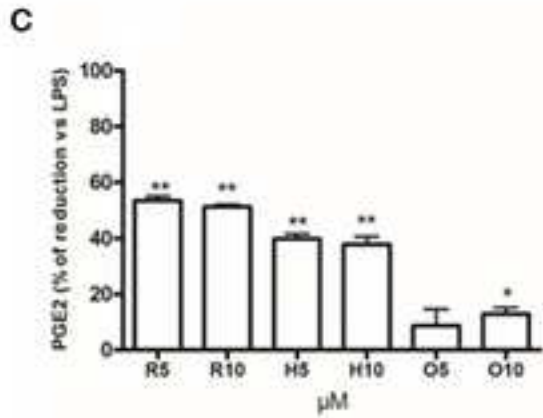
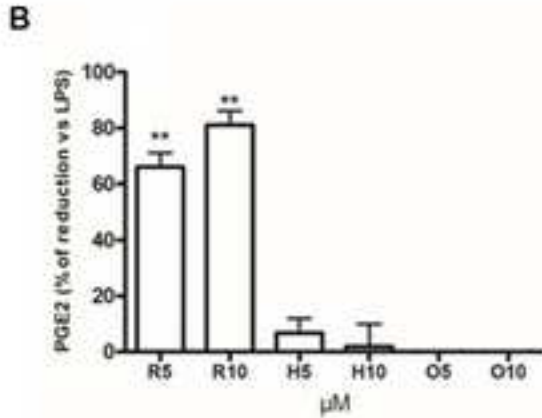
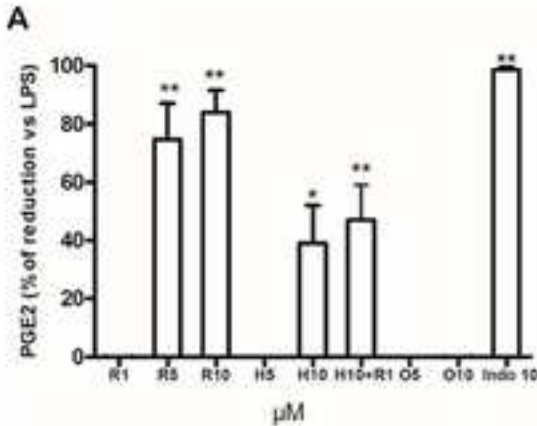
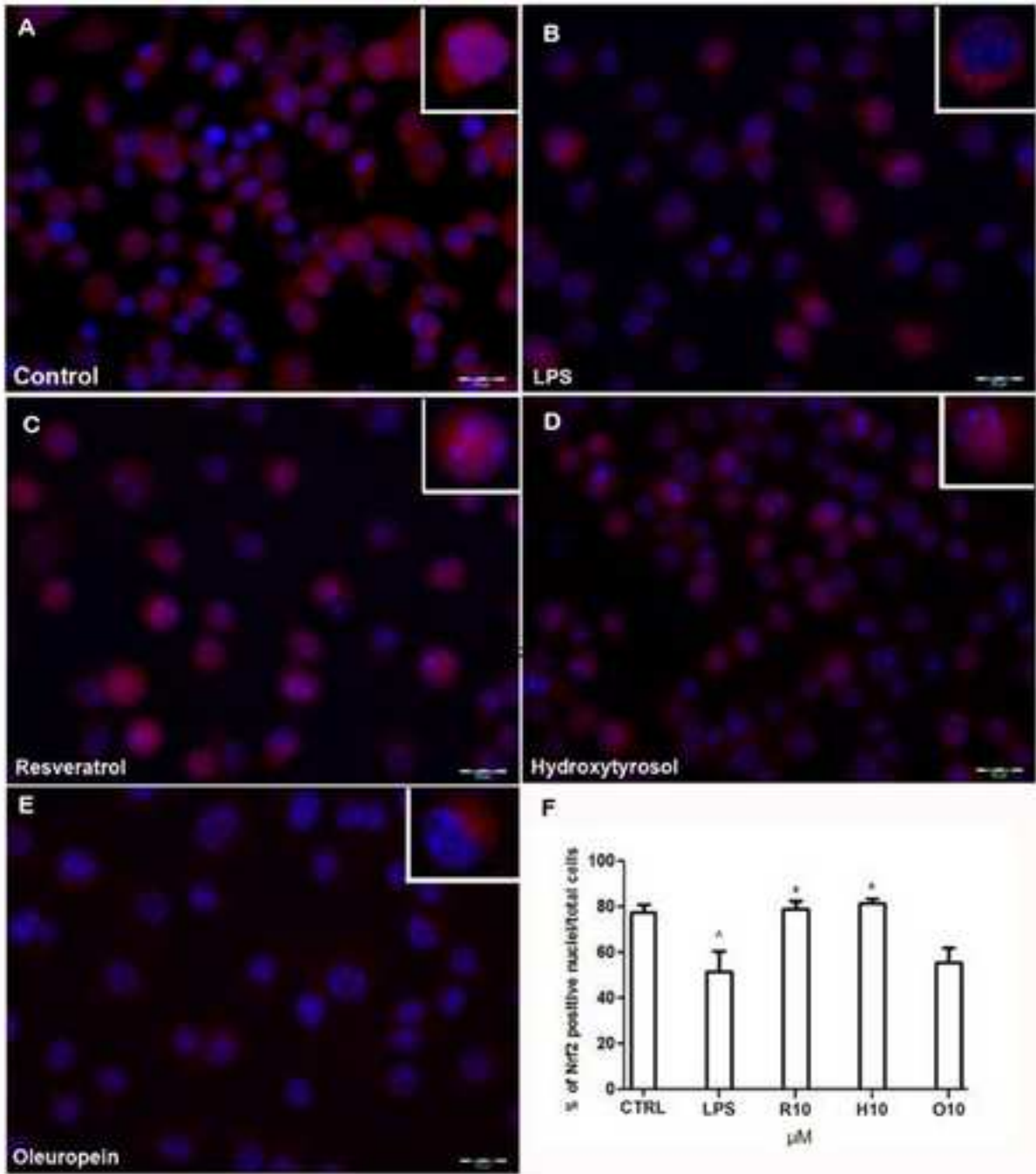


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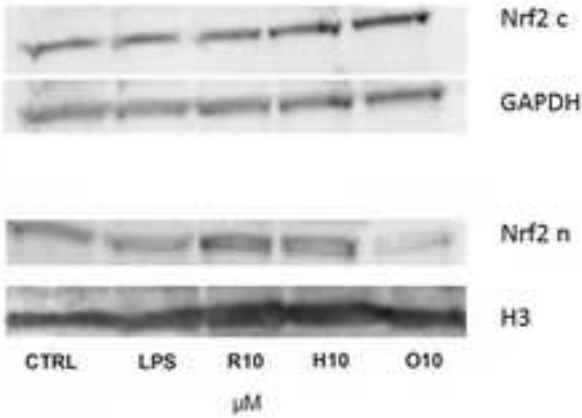
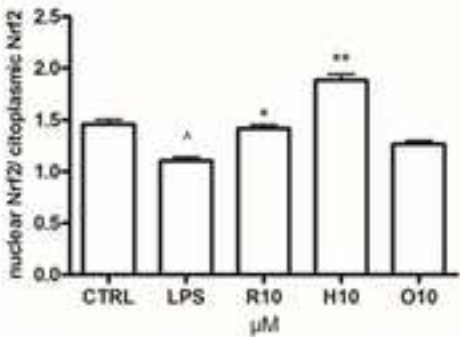
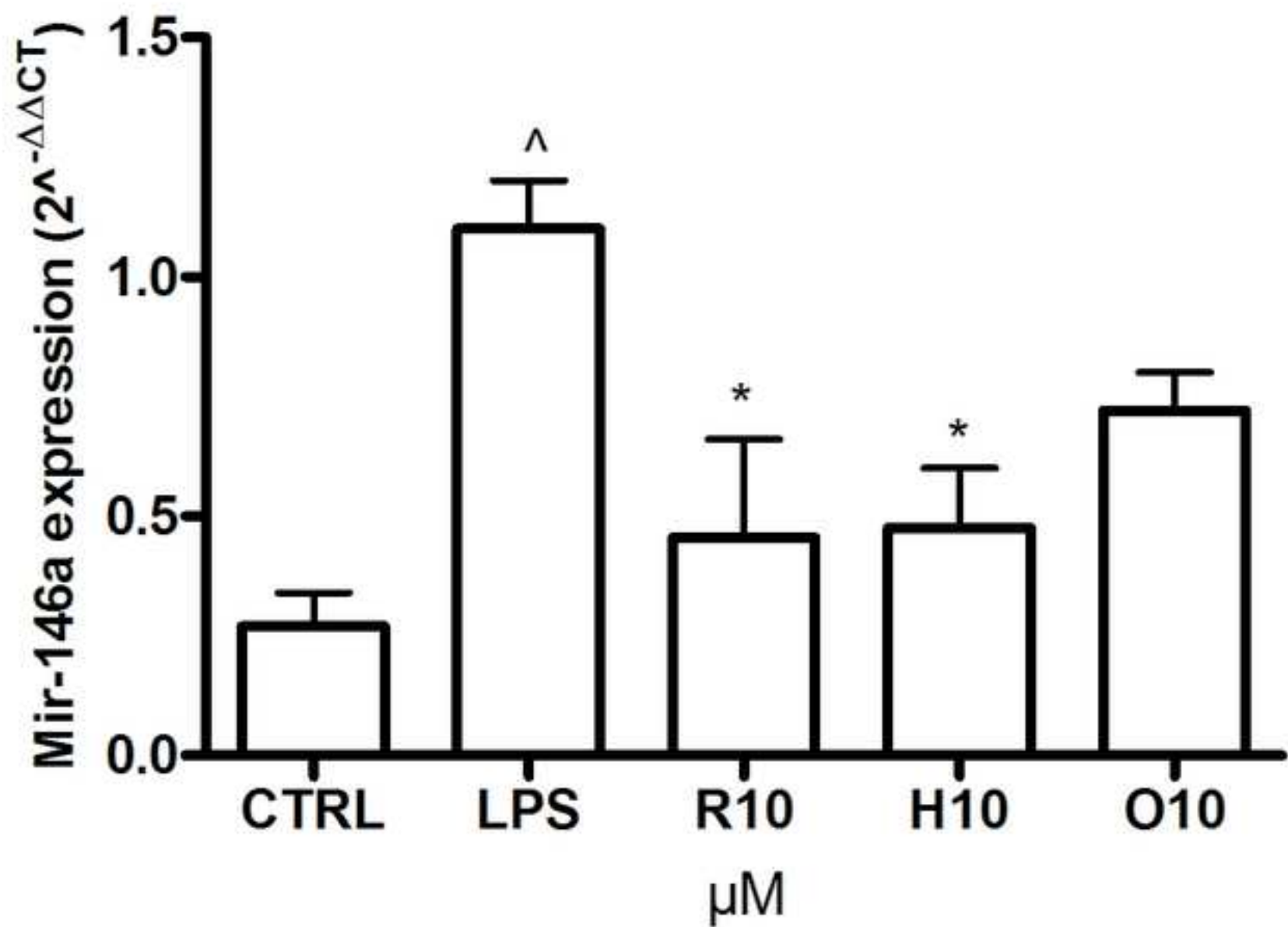


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*Highlights

- Low concentrations of resveratrol and hydroxytyrosol attenuate inflammation
- Resveratrol and hydroxytyrosol mitigate oxidative stress.
- Resveratrol and hydroxytyrosol decrease PGE2 levels.
- miR-146a and Nrf2 are involved in the observed anti-inflammatory effects.